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Adenocarcinoma (MCF-7) Cells by Liposomal Monansin

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13. ABSTRACT (Maximum 200 Words) We have previously shown that stealth monensin liposomes (SML prepared by pH-gradient method) enhances the cytotoxicity of doxorubicin by a factor of 16.5 in MCF-7/dox cells. In the present study, we investigated the effect of SML on the induction of apoptosis and its influence on the expression of multidrug resistance, MDR-1 and multidrug resistance-associated protein, MRP-1 in doxorubicin resistant human breast adenocarcinoma (MCF-7/dox) cells. Apoptosis was studied by TUNEL staining and the expression of multidrug resistance genes (MDR-1 and MRP-1) was elucidated by RT-PCR. It was found that the treatment of MCF7/dox cells with doxorubicin (2.5 µg/ml) with SML (20x10 ⁻⁸ M) combination produced an enhanced apoptotic response (40%), whereas negligible apoptosis was seen in control, doxorubicin (2.5 µg/ml) or SML (20x10 ⁻⁸ M) treated cells. RT-PCR studies showed the expression of both MDR-1 and MRP-1 in MCF-7/dox cells, whereas their expression was negligible in doxorubicin sensitive, MCF-7 cell line. Treatment of MCF7/dox cells to doxorubicin (2.5 µg/ml) enhanced the expression of MDR-1 and MRP-1 by about 50 and 90%, respectively, whereas SML could only produce a limited increase (13-20%) in the MDR genes. On the other hand, the combination treatment of doxorubicin with SML could decrease the doxorubicin induced elevated expression of MDR1 and MRP1 by about 46 and 35%, respectively. Further, the significance of MDR-1 and MRP-1 expression in MCF-7/dox cells was confirmed by the reversal of drug resistance using known MDR-1 inhibitors such as verapamil and cyclosporine and MRP-1 inhibitors such as indomethacin and probenecid.				
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INTRODUCTION

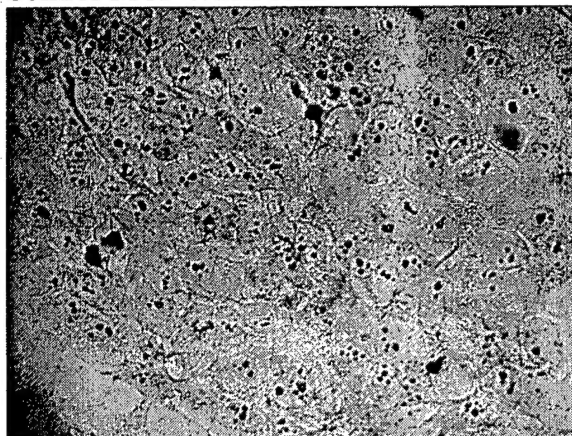
Doxorubicin, a drug of choice for the treatment of breast cancer, has dose limiting cardiotoxicity and its repeated administration may lead to pleiotropic drug resistance in patients. Various agents such as verapamil have been used to overcome the doxorubicin resistance in various clinical studies unsuccessfully. We have previously shown that long-circulating (stealth) monensin liposomes (SML) could enhance the cytotoxicity of anticancer drugs (1,2). In order to increase the entrapment of monensin in SML, we modified our previous method by using pH-gradient method (3) and we also reported that SML prepared by pH-gradient method potentiated the cytotoxicity of doxorubicin, paclitaxel and etoposide in both doxorubicin sensitive and resistant human breast tumor MCF-7 cells (4). The mechanism(s) by which SML enhances the cytotoxicity of doxorubicin in doxorubicin resistant human breast adenocarcinoma, MCF-7/dox cells has not been elucidated yet. Recent data indicate that drug resistance may be attributed to the expression of one or more multidrug resistance genes such as multidrugresistance-Pgp (MDR-1), multidrugresistance-associated protein (MRP-1) and breast cancer resistance protein (BCRP) in MCF-7/dox cells (5-7). We hypothesized that the enhancement of doxorubicin cytotoxicity by SML may be mediated via apoptosis and SML-mediated reduction in the expression of multidrugresistance (MDR) genes. Therefore, the purpose of the present study was to study the effects of SML on apoptosis and expression of MDR-1 and MRP-1 in MCF-7/dox cells. Our data indicate that the combination of SML (20×10^{-8} M) and doxorubicin (2.5 $\mu\text{g/ml}$) produces an enhanced apoptotic response in MCF-7/dox cells, whereas SML or doxorubicin alone had showed negligible apoptosis. Further, our data show that SML could moderately reduce the doxorubicin induced elevated expression of MDR-1 and MRP-1.

BODY

Preparation and characterization of long-circulating (stealth) monensin liposomes (SML) by pH-gradient method: Monensin liposomes were prepared by pH-gradient method and characterized for particle size and entrapment efficiency of monensin as described by Shaik et al 2001 (3).

Effect of SML on the induction of apoptosis in resistant MCF-7 cells: The induction of apoptosis in resistant MCF-7 cells by the combination of 2.5 $\mu\text{g/ml}$ doxorubicin (concentration considerably lower than its IC_{50} value) with non-toxic concentration of SML (20×10^{-8} M) was studied by TUNEL staining. The combination of doxorubicin with SML induced apoptosis in 40% of cells in comparison to less than 10% apoptotic cells observed in control, doxorubicin or SML treated cells (Figure 1).

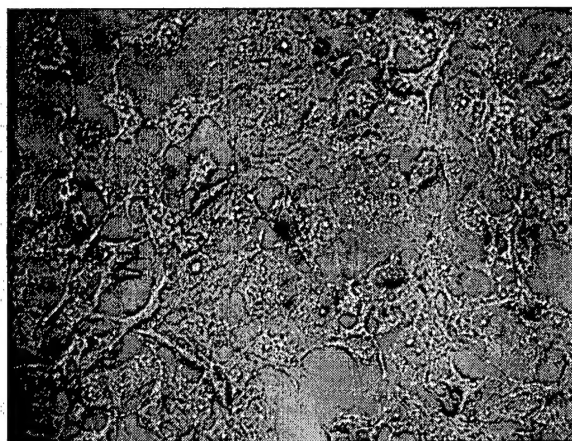
Control-MCF-7/dox cells



SML treated MCF-7/dox cells



DOX (2.5 $\mu\text{g/ml}$) treated MCF-7/dox cells



DOX(2.5 $\mu\text{g/ml}$)+SML treated MCF-7/dox cells

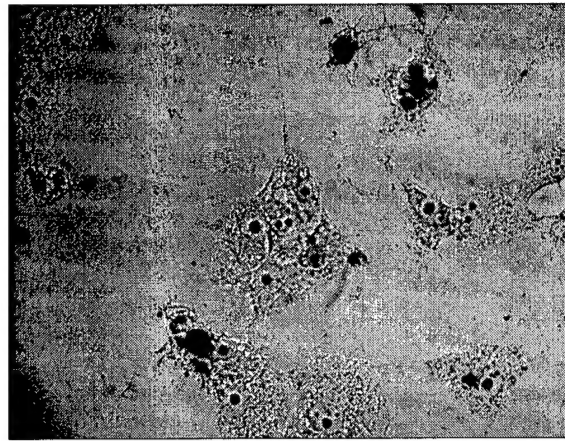


Figure 1: TUNEL staining of MCF-7/dox cells treated with medium, SML, doxorubicin (DOX) or SML with doxorubicin combination.

Effect of SML on MDR1 and MRP1 expression in MCF-7/dox cells: MCF-7/dox cells (1×10^6 in 25 cm^2 flask) were treated with medium, doxorubicin ($2.5 \text{ }\mu\text{g/ml}$), SML ($20 \times 10^{-8} \text{ M}$) or $2.5 \text{ }\mu\text{g/ml}$ doxorubicin with $20 \times 10^{-8} \text{ M}$ SML for 72 hours and the total RNA was eluted using the Eppendorf Perfect RNA Mini Kit (Brinkman Instruments, Westbury, NY). Reverse transcription was performed with Moloney-murine leukemia virus reverse transcriptase (MuLV-RT) (Applied Biosystem, CA, USA) according to the manufacturer's protocol with some modifications. The PCR reaction was performed with MDR-1 (sense, 5' CCA TCA TTG CAA TAG CAG G 3'; antisense, 5' GAG CAT ACA TAT GTT CAA ACT T 3'), MRP-1 (sense 5' GGA CCT GGA CTT CGT TCT CA 3' ; antisense 5' CGT CCA GAC TTC TTC ATC CG 3') and β -actin (sense, 5' GATCATGTTTGAGACCTTC 3'; antisense, 5' GTCAGGCAGCTCGTAG 3') primer pairs and ATAQ DNA polymerase (Applied Biosystem) at 94°C for 2 min, 30 cycles of 94°C , $60^\circ\text{C}/52^\circ\text{C}$ for MDR1/MRP-1 and 72°C (1 min each), and then 10 min at 72°C before holding at 4°C . The 126 bp for MDR-1 and 252 bp for MRP-1 PCR products were separated in a 1.5% agarose gel and the band intensities were normalized with respect to β -actin using Scion Image Software (Beta 3b version, Scion Corporation, Frederick, MD). The results are shown below in Figure 2.

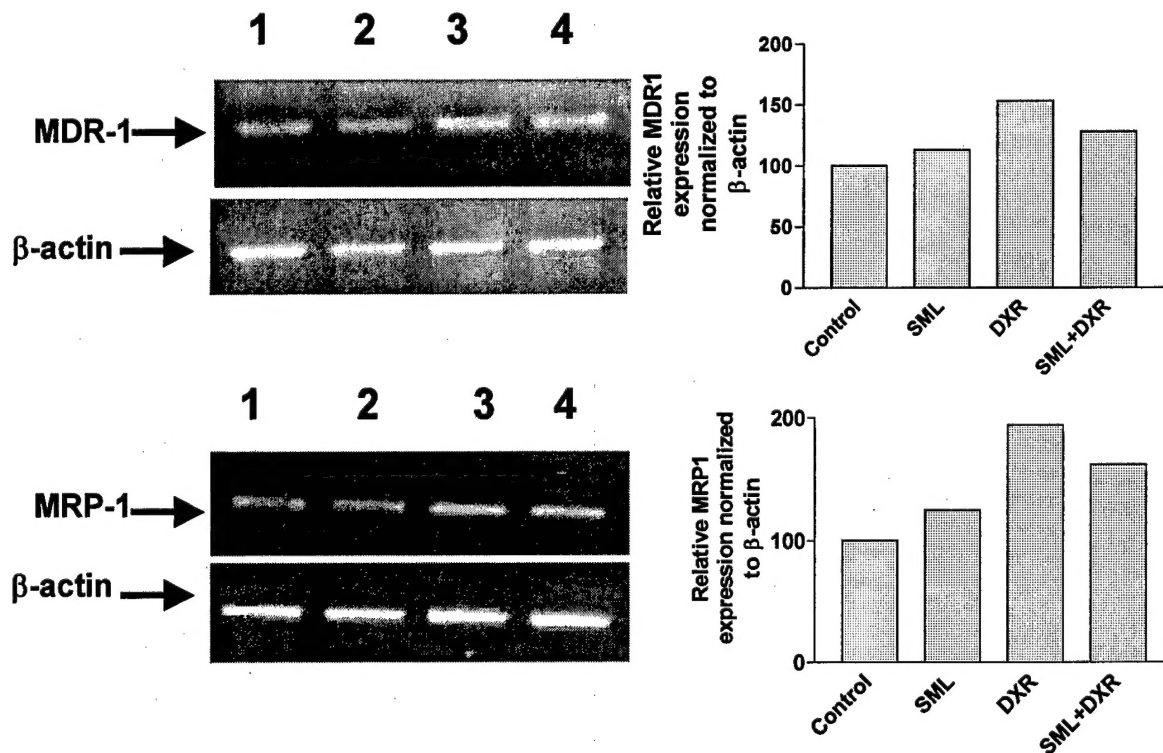


Figure 2: Semi-quantitative RT-PCR analysis of MDR-1 and MRP-1 expression in MCF-7/dox cells. Lane 1-Control cells; Lane 2-SML; Lane-3 Doxorubicin; Lane 4-SML+Doxorubicin. The data were normalized to β -actin expression and such normalized data were used for comparison considering the expression in control cells as 100%.

Effect of MDR-1 and MRP-1 inhibitors on doxorubicin cytotoxicity in MCF-7/dox cells:

The effect of MDR1 inhibitors (cyclosporine and verapamil) and MRP1 inhibitors (indomethacin and probenecid) on the in-vitro cytotoxicity of doxorubicin was studied by the crystal violet dye uptake assay. Briefly, MCF-7/dox cells were seeded at a density of 10,000 cells per well in 96-well plates and incubated overnight. After that the cells were treated with doxorubicin (0.5-30 $\mu\text{g/ml}$) alone, doxorubicin (0.5-20 $\mu\text{g/ml}$) with a known non-toxic concentration of MDR-1/MRP-1 inhibitors (6 $\mu\text{g/ml}$ cyclosporine, 15 $\mu\text{g/ml}$ verapamil, 40 $\mu\text{g/ml}$ indomethacin or 150 $\mu\text{g/ml}$ of probenecid) for 72 hours. The cytotoxicity was assessed by crystal violet assay and expressed with respect to the untreated control.

Table 1: Reversal of doxorubicin resistance in MCF-7/dox cells by SML, MDR-1 inhibitors (cyclosporine and verapamil) and MRP-1 inhibitors (indomethacin and probenecid) as assessed by the ratio of the inhibitory concentration- IC_{50} of doxorubicin alone and to that of the combination of doxorubicin with SML or MDR-1/MRP-1 inhibitors.

Drug treatment	IC_{50} concentration ($\mu\text{g/ml}$)	Drug resistance reversal
Doxorubicin	26.4	0
Doxorubicin with SML	1.6	16.5
Doxorubicin with cyclosporine	1.0	26.4
Doxorubicin with verapamil	4.0	6.6
Doxorubicin with indomethacin	9.0	2.9
Doxorubicin with probenecid	4.5	5.9

SML, cyclosporine, verapamil, indomethacin and probenecid were employed at non-toxic concentrations for the drug resistance reversal studies as described above.

Presently, studies are in progress to determine the effects of the known MDR inhibitors on the MDR-1 and MRP-1 expression and compare the data with that of SML. These studies are aimed at elucidating the molecular mechanisms associated with reversal of doxorubicin resistance by SML in MCF-7/dox cells.

Effect of SML on the in-vivo cytotoxicity enhancement of doxorubicin: In-vivo experiments are now being conducted at the Dr. Agrawal's laboratory at the School of Medicine, Tulane University, New Orleans, LA. Female nu/nu mice (6-8 weeks old) were xenografted with MCF-7 cells and once the tumor reached approximately 100 mm^3 , the mice were given doxorubicin 5 mg/kg i.v on days 3 and 5. Another group of tumor bearing mice were administered with doxorubicin (5 mg/kg) with SML (0.1 ml of 10^{-6} M) and the control mice were given the vehicle. The tumor dimensions were monitored over a period of 8 weeks.

Initial results are not conclusive and further experiments are now being conducted with optimized dosing of doxorubicin and SML.

KEY RESEARCH ACCOMPLISHMENTS

1. The entrapment efficiency of monensin in SML was increased from 2% to 14% by employing pH-gradient method.
2. The SML was successfully freeze-dried with minimal change in particle size upon freeze-drying and subsequent storage, thus improving the physical stability of SML.
3. SML prepared by pH-gradient method were found to overcome the doxorubicin resistance in MCF-7/dox cells.
4. Apoptosis could be induced in MCF-7/dox cells by using non-toxic concentrations of SML with 1/50-1/10 th IC_{50} concentration of doxorubicin.
5. The enhancement of apoptotic response by the combination of SML with doxorubicin was confirmed by multiple techniques such as acridine orange staining, caspase-3 assay and TUNEL staining.
6. Insight into the molecular mechanism associated with drug resistance reversal by SML was made by studying the expression of MDR-1 and MRP-1 in MCF-7/dox cells.
7. RT-PCR studies showed the expression of both MDR-1 and MRP-1 in MCF-7/dox cells and their expression was increased by 50-90% by exposure to doxorubicin. SML were able to decrease the doxorubicin induced elevated expression of MRP-1 and MDR-1 by 35 and 46%, respectively.
8. Current findings suggest that SML do not inhibit the expression of both MDR-1 and MRP-1 in MCF-7/dox cells below its normal levels.

REPORTABLE OUTCOMES

1. **Mandip Singh**, MS Shaik, K. Primus, GA. Salama. Enhancement of the in-vitro cytotoxicity of anticancer drugs against sensitive and resistant human breast tumor MCF7 cells by stealth monensin liposomes. Presented at the 93rd Annual Meeting of the American Association for Cancer Research (AACR) held at San Francisco, CA, April, 2002.
2. **Mandip Singh**, MS Shaik, K. Primus, GA. Salama. Enhancement of the in-vitro cytotoxicity and apoptotic response of doxorubicin in resistant MCF-7 cells by stealth monensin liposomes. Presented at the Era of Hope 2002, Department of Defense Breast Cancer Research Program Meeting held at Orlando, FL, September, 2002.
3. MS Shaik, K. Primus, GA. Salama, Abhijit Chatterjee and **Mandip Singh**. Effects of monensin liposomes on the cytotoxicity, apoptosis and expression of multidrugresistance genes in doxorubicin resistant human breast tumor (MCF-7/dox) cell-line. (Manuscript under preparation).
4. Two different doxorubicin resistant human breast tumor MCF-7 cell lines were developed.
5. Molecular biology techniques such as apoptosis assays, Western blotting and RT-PCR were acquainted.
6. These molecular biology techniques are now routinely used in our laboratory to explore the molecular mechanisms associated with antitumor activities of cyclooxygenase inhibitors.

CONCLUSIONS

Nontoxic concentrations of SML enhance the apoptotic response of doxorubicin in MCF-7/dox cells. The exposure of MCF-7/dox cells to doxorubicin increased the expression of both MDR-1 and MRP-1 genes and such doxorubicin induced multidrug resistance genes could be decreased to a moderate extent by the combination of doxorubicin with SML. The known MDR-1 inhibitors (cyclosporine and verapamil) and MRP-1 inhibitors (indomethacin and probencid) were able to reverse the doxorubicin resistance, thus indicating the existence of both MDR-1 and MRP-1 related drug resistance mechanisms in MCF-7/dox cells.

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#4710 Enhancement of the in-vitro cytotoxicity of anticancer drugs against sensitive and resistant human breast tumor MCF7 cells by stealth monensin liposomes. Mandip S. Sachdeva, Madhu S. Shaik, Kela Primus, and Germain A. Salama. *Florida A&M University, Tallahassee, FL.*

Our laboratory has developed a method for improving the entrapment of monensin in stealth (long-circulating) liposomes by using pH-gradient approach. The stealth monensin liposomes have a particle size of 223 nm, monensin entrapment of 14%, and plasma half-life of 7-8 hr in BALB/c mice. In the present study, the liposomal monensin formulation was studied for its ability to enhance the in-vitro cytotoxicity of doxorubicin (DXR), etoposide (ETP) and paclitaxel (PTX) against both sensitive and resistant MCF7 cells. The cytotoxicity of DXR, ETP and PTX alone and in combination with stealth monensin liposomes was assessed by crystal violet assay. Furthermore, the induction of apoptosis in resistant MCF7 cells by DXR (at 1/50 th IC_{50} concentration) in combination with stealth monensin liposomes was evaluated by acridine orange staining. Our results show that IC_{50} of DXR, ETP and PTX against sensitive MCF7 cells was 0.2 $\mu\text{g/ml}$, 21.0 $\mu\text{g/ml}$ and 0.1 $\mu\text{g/ml}$, respectively. The combination of liposomal monensin (10×10^{-8} M) with DXR, ETP and PTX produced IC_{50} values of 0.04 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$ and 0.002 $\mu\text{g/ml}$ against sensitive MCF7 cells, respectively (a 5, 210 and 50-fold potentiation of DXR, ETP and PTX, respectively). In case of resistant MCF7 cells, the IC_{50} of DXR, ETP, PTX was found to be 26.5 $\mu\text{g/ml}$, 68.0 $\mu\text{g/ml}$ and 5.0 $\mu\text{g/ml}$, respectively and their combination with liposomal monensin (20×10^{-8} M) resulted in IC_{50} values of 1.6 $\mu\text{g/ml}$, 10.9 $\mu\text{g/ml}$ and 1.4 $\mu\text{g/ml}$, respectively (a 16.5, 6 and 3.5-fold potentiation of DXR, ETP and PTX, respectively). Acridine orange staining with resistant MCF 7 cells indicated that apoptosis was induced in at least 30% of cells by using DXR (0.5 $\mu\text{g/ml}$) and liposomal monensin (20×10^{-8} M), as compared to the less than 10% in control, DXR and liposomal monensin treated cells. Our results indicate that it is possible to enhance the cytotoxicity of anticancer drugs like DXR, ETP and PTX by liposomal monensin in both sensitive and resistant MCF7 cells, which may be explored further in cancer chemotherapy.

**ENHANCEMENT OF THE IN VITRO
CYTOTOXICITY AND APOPTOTIC RESPONSE OF
DOXORUBICIN IN RESISTANT MCF7 CELLS BY
STEALTH MONENSIN LIPOSOMES**

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The carboxylic ionophore, monensin has been shown to modulate the doxorubicin resistance in various tumor cell lines in-vitro. However, monensin needs to be formulated in suitable drug delivery system in order to overcome its unfavorable physical and pharmacokinetic properties. We previously developed monensin into long-circulating (stealth) liposomes and showed that it could enhance the in-vitro cytotoxicity of anticancer drugs. In order to increase the entrapment of monensin in liposomes, we modified our previous method by using pH-gradient technique. In the present study, we studied the potential of stealth monensin liposomes (prepared by pH-gradient method) for their effect on the in-vitro cytotoxicity of anticancer drugs (doxorubicin, etoposide, paclitaxel) against both sensitive and resistant human breast tumor MCF7 cells by crystal violet dye uptake assay. Further, the induction of apoptosis in resistant MCF7 cells by the combination of doxorubicin with stealth monensin liposomes was also assessed by acridine orange staining and caspase-3 assay. Our results show that stealth monensin liposomes (10×10^{-8} M) enhance the in-vitro cytotoxicity of doxorubicin, etoposide and paclitaxel against sensitive MCF7 cells by a factor of 5, 261 and 90, respectively. In case of resistant MCF7 cells, there was 16.5, 5.6 and 2.8- fold potentiation of the cytotoxicity of doxorubicin, etoposide and paclitaxel, respectively by monensin liposomes (20×10^{-8} M). There was an enhanced apoptotic response (30%) in resistant MCF7 cells treated with doxorubicin at 0.5 mcg/ml ($1/50$ th IC_{50} concentration for doxorubicin) with nontoxic concentration of monensin liposomes (20×10^{-8} M) in comparison to less than 10% apoptotic response observed in control, doxorubicin and liposomal monensin treated cells. The specific activity of caspase-3 in resistant MCF7 cells treated with doxorubicin (2.5 mcg/ml) and monensin liposomes (20×10^{-8} M) was two times more than that of the cells treated with doxorubicin alone. The results indicate that it is possible to overcome the doxorubicin resistance in MCF7 cells with liposomal monensin, which may be further explored in-vivo in nude mice with human breast tumor xenografts.

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